

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Berenice Y. Reed-Gitomer  
Charles Y.C. Pak

Serial No.: 09/339,352

Filed: June 23, 1999

For: ABSORPTIVE HYPERCALCAURIA  
LOCUS ON CHROMOSOME 1

Group Art Unit: 1653

Examiner: H. Robinson

Atty. Dkt. No.: UTSD:553/MBW

CERTIFICATE OF HAND DELIVERY

I hereby certify that this correspondence is being hand delivered for filing to: Examiner H. Robinson, Group 1653, Commissioner for Patents, Washington, D.C. 20231, on the date below:

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Date

Atina Siddiqui  
Signature

SUBMISSION OF SECOND SUBSTITUTE APPEAL BRIEF

BOX AF

Commissioner of Patents  
Washington, D.C. 20231

Sir:

Appellants hereby submit this Response to the Examiner's oral indication that a Second Substitute Appeal Brief is required. It is believed that no fees under 37 C.F.R. §§ 1.16 to 1.21 are occasioned by the filing of this paper; however, should the Commissioner determine otherwise, the Commissioner is hereby authorized to deduct said fees from Fulbright & Jaworski Deposit Account No. 50-1212/10017634/MBW.

### REMARKS

On March 11, 2002, Examiner Robinson orally indicated to Mark B. Wilson, Appellants' representative, that a Second Substitute Appeal Brief would be required in order to avoid the issuance of a second Notification of Non-Compliance with 37 C.F.R. § 1.192(c). On March 25, 2002, Examiner Robinson confirmed this demand. The grounds for such a demand appear to be manner in which Appellants have identified and referred to the Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer, submitted as Appendix B with attached Exhibit 1, and typographical errors in the text of the brief.

Appellants object to this further, unnecessary delay. Appellants note that a period of four months elapsed between the submission of the original Substitute Appeal Brief and the Examiner's demand for correction of typographical errors.

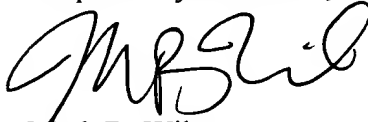
Nevertheless, in view of the additional delays incurred, Appellants have determined that their interest in the expeditious presentation of the appeal leaves them no alternative but to file yet another substitute brief. A Second Substitute Appeal Brief is therefore submitted herewith. Appellants submit that this Second Substitute Appeal Brief is both outside any grounds for rejection of the brief under 37 CFR § 1.192 and MPEP § 1206.

Furthermore, Appellants reiterate here their position that the original Notification of Non-Compliance with 37 C.F.R. § 1.192(c) is defective. Appellants have argued the impropriety of the original Notification of Non-Compliance with 37 C.F.R. § 1.192(c) in a paper entitled "Response to Notification of Non-Compliance with 37 C.F.R. § 1.192(c) and Submission of Substitute Appeal Brief" submitted with the original Substitute Appeal Brief on November 2, 2001. Appellants incorporate the entirety of this paper herein by reference. In summary, Appellants argue that the separate grounds of patentability for claim 2 were presented in the

original brief as filed and that the claims on appeal were identified as required by 37 C.F.R. § 1.192(c)(3).

Entry and consideration of the Second Substitute Appeal Brief is respectfully solicited.

Respectfully submitted,



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Date: March 27, 2002

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PATENT

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Alvin Siddiqui  
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SECOND SUBSTITUTE APPEAL BRIEF

## TABLE OF CONTENTS

I.	REAL PARTY IN INTEREST.....	3
II.	RELATED APPEALS AND INTERFERENCES.....	3
III.	STATUS OF THE CLAIMS .....	3
IV.	STATUS OF AMENDMENTS .....	3
V.	SUMMARY OF THE INVENTION .....	4
VI.	ISSUES ON APPEAL.....	4
VII.	GROUPING OF THE CLAIMS .....	5
VIII.	ARGUMENT .....	5
	A. Claim 1 Has an Asserted Utility.....	5
	1. <i>Asserted Utility Creates Presumption of Utility</i> .....	6
	2. <i>The Asserted Utility Is Understood by Those of Skill in the Art and Does not Require Undue Experimentation</i> .....	7
	B. Claim 1 Is Enabled .....	11
	C. Claim 1 Is Definite .....	13
	D. Claim 2 is Separately Patentable over all Rejections .....	14
	1. <i>Claim 2 Has an Asserted Utility for Additional Reasons</i> .....	14
	2. <i>Claim 2 is Enabled for Additional Reasons</i> .....	15
	E. The Application Should Receive the Provisional Filing Date.....	16
IX.	CONCLUSION.....	16
APPENDIX A:	Claims on Appeal	
APPENDIX B:	Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer	
EXHIBIT 1:	Exhibit 1 of Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer	

**PATENT**

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For: ABSORPTIVE HYPERCALCIURIA  
LOCUS ON CHROMOSOME 1

**SECOND SUBSTITUTE APPEAL BRIEF**

**BOX AF**

Commissioner of Patents  
Washington, D.C. 20231

Sir:

Appellants hereby submit an original and two copies of this Second Substitute Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated November 7, 2000. The Notification of Non-Compliance with 37 C.F.R. 1.192(c) was mailed on October 2, 2001, making the original substitute Appeal Brief due on November 2, 2001. The original substitute Appeal Brief was submitted on November 2, 2001. This Second Substitute Appeal Brief is submitted in response to Examiner Robinson's communications of March 25 and 27, 2002.

The fee for filing the Appeal Brief has been previously paid. Should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, the Commissioner is authorized to deduct said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/10017634/MBW.

**STATEMENT OF SUBSTANCE OF INTERVIEWS**  
**PURSUANT TO 37 C.F.R. § 1.133(b)**

Appellants hereby submits a statement of the substance of the telephone interview with Examiner Robinson on September 27, 2001, pursuant to 37 C.F.R. § 1.133(b). Examiner Robinson telephoned Mark B. Wilson, Appellants' representative, to clarify issues raised in the Appeal brief filed July 16, 2001. Mr. Wilson was unavailable. Mr. Wilson returned the phone call and was informed by Examiner Robinson that the issues were resolved. Mr. Wilson inquired as to what decisions were made based on the response filed. Examiner Robinson informed Mr. Wilson that a Notification of Non-Compliance with 37 C.F.R. § 1.192(c) would be issued. Mr. Wilson indicated that based on the finding of non-compliance he would consider responding with an appeal brief that would set forth why each claim is separately patentable.

Appellants subsequently submitted a Substitute Appeal Brief on November 2, 2001. Subsequent to the submission of the Substitute Appeal Brief, Examiner Robinson orally indicated to Mark B. Wilson, Appellants' representative, that a Second Substitute Appeal Brief would be required in order to avoid the issuance of a second Notification of Non-Compliance with 37 C.F.R. § 1.192(c). The grounds for the Examiner's demand appear to be the manner in which Appellants have identified and referred to the Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer, submitted as Appendix B with attached Exhibit 1, and typographical errors in the text of the brief.

On March 27, 2002, Appellants' representative Thomas M. Boyce telephoned Examiner Robinson to confirm the scope and nature of the objections to the Substitute Brief filed November 2, 2001. Examiner Robinson indicated that all of the grounds for objection under 37 C.F.R. § 1.192(c) listed in the Notification of Non-Compliance dated October 2, 2001 had been overcome. Thus, arguments regarding separate patentability and identification of claims on appeal were satisfactory. Mr. Boyce indicated that a Second Substitute Appeal Brief would be submitted.

#### **I. REAL PARTY IN INTEREST**

The real party in interest is the assignee, Board of Regents, The University of Texas System, Austin, Texas.

#### **II. RELATED APPEALS AND INTERFERENCES**

There are no interferences or appeals for related cases.

#### **III. STATUS OF THE CLAIMS**

Claims 1-26 were originally filed in the present application. During prosecution, claims 8-9, 16 and 18-26 were cancelled and claims 1, 10, 11 and 12 were amended. Accordingly, claims 1-7, 10-15 and 17 are pending. Of these, claims 1-7, 10-15 and 17 are the subject of the present appeal and stand appealed. A copy of the appealed claims is attached as Appendix A to this Brief.

#### **IV. STATUS OF AMENDMENTS**

Appellants have made no amendments subsequent to the final rejection.



## **V. SUMMARY OF THE INVENTION**

This invention involves Appellants' discovery of an area on human chromosome 1 that is genetically linked to hypercalciuria in general and absorptive hypercalciuria in specific. *See* Specification, p. 5, lns. 27-30, p. 6, lns. 1-15. The specific area of chromosome 1 involved in the invention is that region containing 1q23 and 1q24. *See* Specification, p. 6, lns. 12-30. This discovery allows one to screen subjects and determine if the subject has an increased risk of absorptive hypercalciuria. The screening methods generally comprise obtaining a sample of nucleic acid from a subject and analyzing the sample of nucleic acid to detect the presence or absence of a genetic mutation in the genomic region of chromosome 1. The presence of such a mutation indicated in increased risk of hypercalciuria; the absence of such a mutation does not indicate an increased risk. *See* Specification, p. 6, lns. 11-19. The nucleic acid can be analyzed using standard techniques well known and used by those of ordinary skill in the art, for example, PCR, diagnostic RFLP analysis, RNase protection assay or RNase mismatch cleavage.

## **VI. ISSUES ON APPEAL**

The issues for the Board's consideration are:

Whether claims 1-7, 10-15 and 17 are properly rejected under 35 U.S.C. § 101 as lacking a specific and substantial asserted utility or a well established utility.

Whether claims 1-7, 10-15 and 17 are properly rejected under 35 U.S.C. § 112, first paragraph, as lacking a specific utility to enable one skilled in the art to use the claimed invention without undue experimentation.

Whether claims 1-7, 10-15 and 17 are properly rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Appellant regards as the invention.

## **VII. GROUPING OF THE CLAIMS**

For purposes of this Appeal, the claims should stand or fall separately, as described below.

For the purpose of the rejection under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, claim 2 should stand or fall separately from the remainder of the claims. Claim 2 is directed specifically to the determination of whether or not an individual has an increased risk of developing absorptive hypercalciuria, instead of hypercalciuria of any form. The majority of the data in the specification and the Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer, submitted as Appendix B herewith, relates specifically to an increased risk of absorptive hypercalciuria. These data are discussed below. Therefore, although Applicants submit that such a determination would not be supported by the facts of this case, the Board could determine that a claim of the scope of present claim 1 is properly rejected under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, while a claim limited in scope to absorptive hypercalciuria is not properly rejected under these grounds.

## **VIII. ARGUMENT**

### **A. Claim 1 Has an Asserted Utility**

The Action rejects claim 1 under 35 U.S.C. §101 asserting that “the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility based on screening for increased risk of developing hypercalciuria.” The Examiner contends that the specification does not clearly set forth how a standardized screening method would be developed to screen for increased risk of absorptive hypercalciuria (AH). The Examiner also contends that the uncertainty of the gene region and variability of the mutation, may be indicia of a “real world” use, but in view of the absence in the application of working examples and

complete details for carrying out the processes indicated in the claims, the utility indicated would require further experimentation. Appellants traverse this rejection.

*1. Asserted Utility Creates Presumption of Utility*

The Manual of Patent Examining Procedure (MPEP) sets forth the guidelines for compliance with the utility requirement of 35 U.S.C. § 101 in MPEP § 706.3(a)(1). Subsection (B)(1) makes it clear that an invention has utility if a particular purpose (*i.e.* “specific utility”) is asserted by the specification and a person of ordinary skill would consider this assertion credible. Both the Federal Circuit Court and the Court of Customs and Patent Appeals have directed the Patent Office to presume that an Applicant’s assertion of utility is true and is sufficient to satisfy the utility requirement of 35 U.S.C. §101. *See e.g., In re Jolles*, 628 F.2d 1322, 206 USPQ (CCPA 1980); *In re Irons*, 340 F.2d 974, 144 USPQ 351 (CCPA 1965); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA); *In re Sichert*, 556 F.2d 1154, 1159, 196 USPQ 209-212-13 (CCPA 1977).

Appellants have clearly asserted the utility of the claimed invention. The specification at page 6, lines 11-15, states that: “[d]escribed in this invention is a method for screening for an increased risk of hypercalciuria by obtaining a sample nucleic acid from a subject; and analyzing the sample nucleic acid to detect the presence or absence of a genetic mutation in genomic region associated with an increased risk of developing hypercalciuria.” The specific utility asserted by the application is thus the use of the claimed locus in screening for an increased risk of developing hypercalciuria. A person of ordinary skill would find this a credible assertion. The instant invention sets forth a genetic locus that is statistically related to an absorptive hypercalciuria (AH) phenotype in the screened kindred groups. The detection of the altered loci would determine an individual at risk and would facilitate early detection of disease onset and

potential intervention to allow for modifications in lifestyle or diet that could prevent or delay onset of the disease.

Any contention of a “*lack of asserted utility*” simply does not withstand an examination of the facts. The specification clearly discloses a specific genomic region of chromosome 1, 1q23.3-1q24, that is reasonably correlated to a specific disease condition, hypercalciuria, in particular absorptive hypercalciuria (AH). The inventors established a statistically significant linkage between an alteration in this loci and the AH phenotype. The asserted utility of the present invention falls within the guidelines established by the MPEP governing utility of applications. *See* MPEP § 2107.

2. *The Asserted Utility Is Understood by Those of Skill in the Art and Does not Require Undue Experimentation*

A person of ordinary skill will recognize that the instant invention provides substantive evidence that localizes a disease susceptibility phenotype to a specific genetic locus.

Localization of mutations in a genetic locus requires the practice of simple screening processes. Such screening process are well-known to those of skill in the art and are described in great detail in the specification. For example the specification teaches nucleic acid based screening assays at pg. 45, ln. 7, to pg 60, ln. 20. Further, Example 3 of the specification teaches techniques shown by the inventors to allow the determination as to whether there is a mutation in the 1q23.3-1q24 region, at pg. 124, ln. 20, to pg. 126, ln. 7. Example 4 of the specification teaches a typical technique that may be used to identify individuals at risk from hypercalciuria, at pg. 126, ln. 9, to pg. 127, ln. 19. Example 5 describes some specific genetic mutations that are linked to hypercalciuria, which were determined using the techniques disclosed in the specification, pg. 127, ln. 21, to pg. 128, ln. 28.

In addition to the data contained in the specification, which was available at the time the application was filed, the Inventors have now obtained even more data that support that mutations in the 1q23.3-1q24 region are indicative of an increased risk for hypercalciuria. Attached as Appendix B is a Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer. Drs. Pak and Reed-Gitomer are the inventors of this application. Appellants note that a typographical error present in ¶11 of the original declaration has been corrected. The erroneous reference to Table 1 has been corrected to refer to Table 5. Drs. Pak and Reed-Gitomer have therefore re-executed the declaration after correction of the error.

In their Declaration, Drs. Pak and Reed-Gitomer set forth that the present claims are based on the inventors' discovery that an area on human chromosome 1, 1q23.3-1q24 is linked to absorptive hypercalciuria (AH). The claims relate to methods allowing for the determination as to whether an individual has a genetic predisposition for hypercalciuria. Appendix B, ¶3. They set forth known techniques described in the specification that may be used to screen for mutations in the 1q23.3-1q24 region. For example, they cite to pg. 45, ln. 7, to pg 60, ln. 20, pg. 124, ln. 20, to pg. 126, ln. 7, pg. 126, ln. 9, to pg. 127, ln. 19, and pg. 127, ln. 21, to pg. 128, ln. 28 of the specification for the teaching of such techniques, their use in the context of the invention, and results supporting the utility of the claimed methods. Appendix B, ¶4.

With their Declaration, the inventors submit new data which have been obtained with these techniques. These data confirm that the presence of a mutation in one or both alleles of 1q23.3-1q24 is associated with a significant increase in estimated risk for the occurrence of the AH phenotype. The new data are included and discussed in detail in a manuscript attached to the Declaration as Exhibit 1. Appendix B, ¶5.

These new data are even further evidence that the claimed methods of analyzing sample nucleic acid to detect the presence or absence of a genetic mutation in the genomic region of 1q23.3-1q24 allow for the determination of whether one has a genetic predisposition for AH. Specifically, the data show that screening of genomic DNA from 16 subjects revealed 6 base changes in 1q23.3-1q24. Four of six base changes found in 1q23.3-1q24 were shown to indicate a significant increase in the relative risk for AH. For one of the base changes, it was not possible to calculate a risk odds ratio, because the control population was found to contain no mutant alleles. One base change was found to have a non-significant odds ratio. Appendix B, ¶¶6-8.

In view of these new data, the apparent suggestion of the Action that the claimed invention lacks utility because it is not possible to know which mutations that may be found in the 1q23.3-1q24 region are linked to an increased risk of hypercalciuria is simply not the case. As shown in the data in the specification and the Declaration of Drs. Pak and Reed-Gitomer, the occurrence of mutations in the 1q23.3-1q24 region is correlated with the increased risk of hypercalciuria. This correlation does not appear limited to a single or small subset of mutations. In fact, most such mutations thus far found have been found to be indicative in a significantly increased risk of AH. Appendix B, ¶9.

The apparent suggestion of the Action that the invention only has utility for and is only enabled in regard to the detection of specific mutations listed in the specification is not correct. Rather, Applicants have shown that having a mutation in the 1q23.3-1q24 region is statistically correlated to an increased risk of hypercalciuria. The present invention is not limited to cases where a subject is diagnosed as positively having hypercalciuria. Rather, it encompasses all instances where one screens for a mutation in the 1q23.3-1q24 region, and thereby, determines whether or not the subject has a increased risk of developing hypercalciuria

Based on the data in the specification and their Declaration, the Applicants have declared that, “[b]y following the teachings of the specification and employing the methods taught in the specification and known to those of skill in molecular biology, skilled molecular biologists will recognize that they can practice the claimed invention and determine whether a subject has a mutation in the 1q23.3-1q24 region, and thereby, determine whether or not the subject has a increased risk of developing hypercalciuria.” Appendix B, ¶10. Drs. Pak and Reed-Gitomer further state that, in view of the data in the specification and the additional data supplied in Table 5, the invention as claimed has utility and is enabled by the specification such that one of skill in the art would know how to use the present invention. Appendix B, ¶11.

Therefore it would not require undue experimentation to perform similar screening on individuals at risk for AH using methods similar to those set forth in the specification but restricted to the claimed loci. In its simplest embodiment, the instant invention may be carried out by performing screening procedures similar to those used for detecting the initial loci. In addition, a person of ordinary skill would be aware of more refined methods to screen for chromosomal deletions, alterations or other mutations.

The Examiner’s mere contention that it would be undue experimentation due to the absence of data is merely speculation by the Examiner. The Examiner appears to assert more knowledge than the skilled artisans and thus, is substituting her judgment for that of an established expert in the art. This is improper. *In re Zeidler*, 682 F.2d.961, 966-967 (Fed. Cir. 1982). Appellants assert that the Office must provide specific, evidentiary scientific basis (e.g., scientific journal articles, or excerpts from patents or scientific treatises) for its factual conclusions that the present invention “lacks an asserted utility”.

Therefore, in view of the arguments that one of skill in the art would reasonably recognize a correlation between the asserted utility and the ability to screen for AH as the instant specification sets forth, Appellants respectfully request that the Board overturn the rejection of the claims for “lack of utility.”

**B. Claim 1 Is Enabled**

The Action rejects claim 1 under 35 U.S.C. 112, first paragraph, asserting that “the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility” and thus a person of ordinary skill would not know how to use the invention without undue experimentation. In the Action, the Examiner also reinstated her contentions that the specification is not enabled for one skilled in the art to make and use the claimed invention. The Action contends that the claimed invention is enabled for the nucleic acid sequence of SEQ ID NO: 1 that encodes a protein contained in SEQ ID NO:2, however, does not provide enablement for any hypercalciuria gene nor a screening method for “increased risk” of AH. Appellants respectfully traverse this rejection.

As set forth above, the specification sets forth a screening method useful in detecting individuals potentially at risk for the development of hypercalciuria or a related phenotype. Thus, one of skill in the art would understand the utility of the invention as claimed.

A rejection based on a lack of enablement must be adequately supported by substantive evidence. The PTO is required to assume that the specification complies with the enablement provisions of Section 112 unless it has “acceptable evidence or reasoning” to suggest otherwise. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 USPQ 367, 369-370 (CCPA 1971). The PTO must therefore provide reasons supported by the record as a whole of what the specification is not enabling. *Application of Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219-220 (CCPA 1979).



Then and only then does the burden shift to the applicant to show that one of ordinary skill in the art could have practiced the claimed invention without undue experimentation. *In re Strahilevitz*, 668 F.2d. 1229, 1232, 212 USPQ 561, 563-64 (CCPA 1982). [Emphasis added]

The Action has erroneously placed the burden of proof on the Appellants without offering any evidence or reasoning based on the record as a whole why the disclosure is not enabling for the pending claims. The single “grounds” of rejection is without support and is couched in terms that a person of ordinary skill would not be enabled to carry out the invention because one of ordinary skill would have no way of recognizing a utility for the invention. This is wrong.

The instant specification sets forth a means for screening for hypercalciuria. Linkage analysis was performed in order to establish the correlation between the AH phenotype and the disclosed loci. A person of ordinary skill would understand that similar means to those taught by the specification could be employed to screen for the altered loci in other individuals deemed to be at risk for the development of the disease, *i.e.*, based on family history, as the specification teaches a successful means of screening (*see* examples 1 and 2). If the rejection is to be maintained under §112, the Examiner must support the noted position by citing published references or by Examiner’s Affidavit, as required by MPEP 2144.03.

No undue experimentation is needed to practice the invention, because Appellants’ disclosure clearly enables a screening method for determining an individual with an “increased risk” of the AH phenotype. A person of ordinary skill could readily develop methods of screening for defects within the disclosed region.

Appellants provide sufficient evidence that inherited hypercalciuria is, in some individuals, linked to an inherited defect in the 1q23.3-1q24 region of chromosome 1. *See*

Specification, page 130, Table 7. The Appellants successfully established, through linkage analysis, that a genetic defect (*e.g.*, specific mutations) exhibited by three unrelated, effected kindred localized to the q arm of chromosome 1 at 1q23.3q-q24. Based upon this information, it would not require undue experimentation to derive a means of screening individuals for an increased risk of AH based upon a similar genetic defect. Techniques would clearly be within the purview of a person of ordinary skill to readily develop screening techniques based upon this disclosure for determining whether individuals are genetically predisposed to developing AH. Further, the specification provides teaching related to how such methods could be derived including means of eliciting specific defects within the region (*See*, for example, pg. 58, ln. 20 *et. seq*) as well as sequence information for areas within the disclosed region (*See*, for example, SEQ ID NOs. 1-11). One of ordinary skill would recognize that the disclosure provides adequate information such that assays could be developed employing techniques to detect specific inherited defects indicative of a genetic predisposition for AH (for example, RNase protection or DGGE as discussed in the specification at pg. 58 ln. 20 *et. seq.*).

Appellants do not appreciate that the Action asserts that the working example of Example 5 (*See* Specification, pages 127-131) is inadequate. An application need not include working examples in order to be enabled. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA). The fact that the Application provides a working example that correlates specific mutations located in the chromosome 1q24 locus to the AH phenotype should not be dismissed for being inadequate. Based upon the knowledge of one of ordinary skill in the relevant art and the guidance provided in the present Application, it would not require undue experimentation to use the loci and sequence information provided by the Appellants to screen for specific defects

within the 1q23.3q-q24 region of chromosome 1 and correlate the presence of a genetic defect as a predictor of increased risk for the AH phenotype.

Further, as discussed above, the inventors have submitted, in the form of a declaration, new data that even further establish the utility and enablement of the invention.

In view of the above, Appellants request that the Board overturn the enablement rejection.

### **C. Claim 1 Is Definite**

The Action rejects claim 1 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the Appellants regard as the invention. Claim 1 is deemed indefinite by the Action because “species (a) does not define what mutation is being detected [and] [f]urthermore, it is unclear how species (b) relates to an increased risk.” The Action further finds the claim indefinite because of the amendatory language previously submitted and because it is not demonstrated where the mutation causes increased risk. Appellants respectfully traverse.

Appellants submit that the standard being applied by the Action is improper. In order to satisfy the requirements of definiteness under §112, a claim, read in light of the specification, must reasonably apprise those of skill in the art of its scope. *See Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 927 F.2d 1200, 1217, 18 USPQ2d 1016, 1030 (Fed.Cir.). The specification sets forth a standard means for successfully detecting the altered loci linked to the hypercalciuria susceptibility phenotype, the specific mutated sequence is not necessarily relevant to the elucidation of an indicator of hypercalciuria. Linkage analysis successfully determined an altered loci linked to hypercalciuria. This, in and of itself, has utility in screening for hypercalciuria susceptibility. While a “mutation” is obviously the basis of the detected

chromosomal alteration, elucidation of the specific nucleotide change is not, at this point, necessarily pertinent to the ability to successfully screen for the change. Nevertheless, the screening method that successfully detected the chromosomal change by linkage analysis is a "mutation" and thus the term is employed properly in the claim.

The Action apparently seeks to require that the specification set forth the exact mutated sequence that leads to the development of hypercalciuria. This requirement is, however, not the threshold for patentability and is unnecessary to properly execute the invention within the scope of the claim. The specification sets forth a basic methodology for detecting the altered loci. One of ordinary skill would further recognize that a variety of commonly practiced screening procedures could be carried out to detect hypercalciuria susceptibility based upon the recognition of the significance of the disclosed loci. Furthermore, based upon the derivation of the role of the disclosed loci, it would not require undue experimentation nor any inventive input to detect specific mutated sequences within the disclosed loci that might further relate to the development of hypercalciuria. This is demonstrated by the new data submitted in the Declaration of Drs. Pak and Reed-Gitomer, which confirm that the methods of the specification are useful to detect a risk of hypercalciuria.

In light of the above arguments, the Board should overturn this rejection.

**D. Claim 2 is Separately Patentable over all Rejections**

*1. Claim 2 Has an Asserted Utility for Additional Reasons*

In addition to the above arguments, which apply to all of the claims, Applicants further assert that the subject matter of current claim 2 has utility for even additional reasons. Claim 2 is directed specifically to determining whether or not one has an increased risk of absorptive hypercalciuria, the specific form of hypercalciuria about which the inventors have gathered the

most information. Although Appellants submit that they have shown that the invention has utility in regard to determining whether or not one has an increased risk of hypercalciuria in general, it is possible that the Board could disagree with this broad of a utility. However, Appellants submit that there are additional and compelling arguments for the utility of the subject matter of claim 2, since all of the mutations which have been found to result in hypercalciuria have also been specifically linked to absorptive hypercalciuria. Therefore, even if the Board does not find the subject matter of claim 1 to have utility, the subject matter of claim 2 should be found to have utility.

In view of the above, Appellants request that the Board overturn the utility rejection for claim 2.

2. *Claim 2 is Enabled for Additional Reasons*

In addition to the above arguments, which apply to all of the claims, Applicants further assert that the subject matter of current claim 2 is enabled for even additional reasons. Claim 2 is directed specifically to determining whether or not one has an increased risk of absorptive hypercalciuria, the specific form of hypercalciuria about which the inventors have gathered the most information. Although Appellants submit that they have shown that the invention is enabled in regard to determining whether or not one has an increased risk of hypercalciuria in general, it is possible that the Board could disagree with this broad of enablement. However, Appellants submit that there are additional and compelling arguments for the enablement of the subject matter of claim 2, since all of the mutations which have been found to result in hypercalciuria have also been specifically linked to absorptive hypercalciuria. Therefore, even if the Board does not find the subject matter of claim 1 to be enabled, the subject matter of claim 2 should be found to be enabled.

In view of the above, Appellants request that the Board overturn the enablement rejection for claim 2.

**E. The Application Should Receive the Provisional Filing Date**

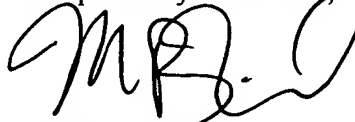
The Action asserts that the Appellants have “not complied with one or more conditions for receiving the benefit of an earlier filing date.” The Action contends that the application will be provided with the filing date of the present application because “the Examiner read the provisional application and did not find support for sequences recited in the claims and disclosed in the specification” and “the marker D1S2681 wherein the genomic region of the invention is comprised.” Appellants respectfully traverse this allegation.

Appellants note that, as no prior art has been cited against the instant application, receiving the provisional filing date is not relevant to the present appeal. Nevertheless, Appellants submit that, while additional material was provided when the provisional application was converted to the instant application, it is nevertheless improper to assert that the full scope of the claims as currently pending were not taught by the original provisional application. Claim scope that is enabled by the initial provisional application should be accorded the earlier filing date under the requirements of 35 U.S.C. § 119(e). For example, claim 1 includes the limitation “said genomic region is comprised in chromosome 1q23.3-1q24” but does not limit the loci to D1S2681. The chromosomal region 1q23.3-1q24 as claimed is properly designated in the provisional application. Therefore, as the claim scope applies to this limitation, the earlier filing date is proper.

## IX. CONCLUSION

Appellants have provided arguments that overcome the pending rejections. Appellant respectfully submits that the Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Respectfully submitted,



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Date: March 27, 2002

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## **APPENDIX A: Claims on Appeal**

1. A method for screening for an increased risk of hypercalciuria comprising:
  - (a) obtaining a sample nucleic acid from a subject; and
  - (b) analyzing the sample nucleic acid to detect the presence or absence of a genetic mutation in a genomic region associated with an increased risk of developing hypercalciuria, wherein said genomic region is comprised in chromosome 1q23.3-1q24.
2. The method of claim 1, wherein the hypercalciuria is further defined as absorptive hypercalciuria.
3. The method of claim 1, wherein the hypercalciuria is further defined as osteoporosis with hypercalciuria.
4. The method of claim 3, wherein the osteoporosis with hypercalciuria is further defined as idiopathic osteoporosis with hypercalciuria.
5. The method of claim 3, wherein the osteoporosis with hypercalciuria is further defined as postmenopausal osteoporosis with hypercalciuria.
6. The method of claim 1, wherein the nucleic acid is DNA.
7. The method of claim 1, wherein the subject is a human.
10. The method of claim 1, wherein the genomic region is located between markers D1S2681 and D1S2815.
11. The method of claim 1, wherein the genomic region has a sequence contained in SEQ ID NO:1.

12. The method of claim 1, wherein the genomic region has a sequence contained in at least one genetic sequence selected from the group consisting of the genetic sequences set forth in GenBank Accession # Z97876, (SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9), GenBank Accession # Z99943 (SEQ ID NO: 10), and GenBank Accession # AL031733 (SEQ ID NO: 7).

13. The method of claim 1, wherein the genomic region has a lod score of greater than 3.0 but less than 30.0.

14. The method of claim 1, wherein analyzing the sample nucleic acid is done with a PCR procedure, diagnostic RFLP analysis, RNase protection assay, or RNase mismatch cleavage assay.

15. The method of claim 14, wherein analyzing the sample nucleic acid is done with a PCR procedure.

17. The method of claim 15, wherein the screening for an increased risk of hypercalciuria comprises:

- (a) obtaining a sample nucleic acid from a subject; and
- (b) analyzing the sample nucleic acid to detect the presence or absence of a genetic mutation in genomic region associated with an increased risk of developing hypercalciuria.

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**APPENDIX B: Declaration of Charles Y.C. Pak and Berenice Y. Reed-Gitomer**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Berenice Y. Reed-Gitomer

Charles Y.C. Pak

Group Art Unit: 1653

Serial No.: 09/339,352

Examiner: H. Robinson

Filed: June 23, 1999

Atty. Dkt. No.: UTSD:553/MBW

For: ABSORPTIVE HYPERCALCIURIA  
LOCUS ON CHROMOSOME 1

**DECLARATION OF CHARLES Y.C. PAK AND  
BERENICE Y. REED-GITOMER UNDER 37 U.S.C. § 1.132**

We, Charles Y.C. Pak and Berenice Y. Reed-Gitomer, do hereby declare and state the following:

1. We are both professors at the Center for Mineral Metabolism & Clinical Research at the University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, and inventors of the above referenced application.
2. We have read the above-captioned patent application, as well as the Official Actions and Responses to Official Actions in this case.
3. The present claims are based on our discovery that an area on human chromosome 1, specifically that area including 1q23.3 and 1q24, is linked to absorptive hypercalciuria ("AH"). The claims relate to methods allowing for the determination as to whether an individual has a genetic predisposition for hypercalciuria. The claimed methods comprise obtaining a sample of nucleic acid from a subject and analyzing the sample of nucleic acid to detect the presence or absence of a genetic mutation in the

genomic region of 1q23.3 and 1q24. The data obtained from the screening methods can be analyzed using standard statistical calculations to determine an increased risk of the AH phenotype.

4. Known techniques may be used to screen for mutations in the 1q23.3-1q24 region. These techniques are described in the patent specification. For example, the specification teaches nucleic acid based screening assays at pg. 45, ln. 7, to pg 60, ln. 20. Further, Example 3 of the specification teaches techniques that allow the determination as to whether there is a mutation in the 1q23.3-1q24 region, at pg. 124, ln. 20, to pg. 126, ln.

7. Example 4 of the specification teaches a typical technique that we have used to identify individuals at risk from hypercalciuria, at pg. 126, ln. 9, to pg. 127, ln. 19. Example 5 describes some specific genetic mutations that are linked to hypercalciuria, which were determined using techniques disclosed in the specification, pg. 127, ln. 21, to pg. 128, ln. 28. This is confirmed by additional data we have obtained, which is discussed below.

5. Since the time of filing of the application, these techniques, were recently used to screen additional individuals for the presence or absence of mutations in 1q23.3-1q24 and determine the increased risk of AH occurrence. The presence of a mutation in one or both alleles of 1q23.3-1q24 was associated with a significant increase in estimated risk for the occurrence of the AH phenotype. The new data supporting this are included in the manuscript attached hereto as Exhibit 1.

6. The data in Exhibit 1 are even further evidence that the claimed methods of analyzing sample nucleic acid to detect the presence or absence of a genetic mutation in

the genomic region of 1q23.3-1q24 allow for the determination of whether one has a genetic predisposition for AH.

7. Exhibit 1 reports that screening of genomic DNA from 16 subjects revealed 6 base changes in 1q23.3-1q24.

8. As shown in Table 5 of Exhibit 1 and discussed in the text of Exhibit 1, four of six base changes found in 1q23.3-1q24 were shown to indicate a significant increase in the relative risk for AH. For one of the base changes, it was not possible to calculate a risk odds ratio, because the control population was found to contain no mutant alleles. One base change was found to have a non-significant odds ratio.

9. We understand that the Examiner in charge of this case has suggested that it is not possible to know which mutations that may be found in the 1q23.3-1q24 region are linked to an increased risk of hypercalciuria. This is not the case. As shown in the data in the specification and submitted herewith, the occurrence of mutations in the 1q23.3-1q24 region is correlated with the increased risk of hypercalciuria. This correlation does not appear limited to a single or small subset of mutations. In fact, most such mutations thus far found have been found to be indicative in a significantly increased risk of AH.

10. By following the teachings of the specification and employing the methods taught in the specification and known to those of skill in molecular biology, one can practice the claimed invention and determine whether a subject has a mutation in the 1q23.3-1q24 region, and thereby, determine whether or not the subject has an increased risk of developing hypercalciuria.

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11. In view of the data in the specification and the additional data supplied in Table S, the invention as claimed can be used to determine whether one has an increased risk of hypercalciuria and, further, the specification describes how to use the present invention to one skilled in molecular biology.

12. In view of the data in the specification and this declaration, skilled molecular biologists will recognize the utility of the presently claimed invention to determine whether one has an increased risk of hypercalciuria.

13. We hereby declare that all statements made herein on our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

March 27, 2002

Date

March 27, 2002

Date

Charles Y.C. Pak

Charles Y.C. Pak

Bernice V. Reed-Gitomer

Bernice V. Reed-Gitomer

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# EXHIBIT 1



**EXHIBIT 1 of Pak and Reed-Gitomer Declaration:**

**Manuscript: "Analysis of the Human Absorptive Hypercalciuria Gene and Association of Base Changes with Low Spinal Bone Density" by Berenice Y. Reed, William L. Gitomer, Howard J. Heller, Ming Chue Hsu, Martha Lemke, Paulette Padalino and Charles Y.C. Pak., with figures and confirmation of the electronic GenBank submission via email on December 21, 2000.**

**Analysis of the Human Absorptive Hypercalciuria Gene and Association of Base Changes  
with Low Spinal Bone Density**

**Berenice Y. Reed, William L. Gitomer, Howard J. Heller, Ming Chue Hsu,  
Martha Lemke, Paulette Padalino and Charles Y.C. Pak**

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**This work was supported by USPHS grants PO1-DK20543 and MO1-RR00633 and the Robert  
T. Hayes Center for Mineral Metabolism Research.**

**Running Title: Gene mutation in AH**

**Mesh Words: nephrocalcinosis, mutation, calcium, bone density, adenylate cyclase.**

## Abstract

Absorptive hypercalciuria (AH) is a common kidney stone forming condition frequently complicated by bone loss. Prior linkage analysis localized the AH gene defect to chromosome 1q23.3-q24. In this report we describe sequence analysis of the 5085 base pair transcript of the human intestinal "AH gene" which maps to this locus. Preliminary screening of genomic DNA from peripheral blood lymphocytes from 16 Caucasian patients with AH revealed 4 polymorphisms and 2 mutations in the AH gene. Of these, two polymorphisms and 1 mutation were identified in the probands from three kindreds, who were subjects of the original linkage study. The frequencies of occurrence of these and the other base changes found were evaluated in the DNA from 80 unrelated AH patients and 132 healthy normal individuals. All base changes occurred with increased frequency in the AH population. Calculated odds ratios for each genotype showed that the presence of an individual base change was associated with a 2.3 to 3.2-fold increase in estimated risk for AH ( $p < 0.02$ ). In addition, the presence of one or more base changes was associated with a lower vertebral bone density. This is the first description of a specific gene defect linked to AH.

## Introduction

Absorptive hypercalciuria (AH) is a common cause of calcium oxalate nephrolithiasis (1). Biochemically, AH is characterized by intestinal hyperabsorption of calcium in the presence of normal serum calcium and immunoreactive parathyroid hormone (iPTH) (2-4). It is often accompanied by low bone mineral density (BMD), particularly of the lumbar spine (5-7). About one-half of patients with AH present with a family history of calcium oxalate nephrolithiasis and hypercalciuria. This high familial transmission of the disease indicates that AH is, at least in part, genetic in origin (8). Thus, an autosomal dominant pattern of inheritance has been reported in some families with the AH phenotype (9-10). However, the nature of the genetic defect(s) in AH has not previously been reported.

In an earlier study, we conducted linkage analysis in three well-characterized and phenotypically similar families with AH. The phenotypic presentation of intestinal hyperabsorption of calcium and hypercalciuria was mapped to chromosome 1q23-24 (11). The region defined by our linkage studies contained 25 known genes and 24 new genes based on information from the human genome project (<http://www.ncbi.nlm.nih.gov>). None of the known genes were likely candidates for the AH defect. Among new genes, one encoded a hypothetical protein (Gen Bank accession AL035122), and was chosen as the initial target in our search for the putative gene in AH, henceforth to be called the "AH gene".

The present study was undertaken to elucidate the structure of the AH gene and to ascertain whether the presence of mutations or polymorphisms in this gene might be associated with low spinal BMD.

## Materials and Methods

**Study Subjects and Evaluation.** All subjects gave informed consent to participate in the protocol that was approved by the Institutional Review Board of UT Southwestern Medical Center at Dallas. For the purpose of the genetic analyses, study subjects were drawn from the Caucasian population in order to eliminate confounding factors due to ethnic variation.

Clinical evaluation of AH patients. Eighty unrelated patients with AH were identified from our kidney stone clinic. The diagnosis of AH was made after either inpatient or outpatient evaluation.

For the inpatient evaluation, patients were admitted to the General Clinical Research Center for 4 days where they were maintained on a constant metabolic diet containing 100 mmol sodium, 10 mmol calcium and 26 mmol phosphorous per day for the first 3 days (Days 1-3). They were kept on an instructed diet of similar composition for 1 week prior to admission. Fasting venous serum samples were drawn on days 1 - 4 and were analyzed for calcium and alkaline phosphatase (Smith-Kline Beecham, Dallas, TX). Fasting venous serum samples on days 1 and 4 were also analyzed for iPTH by immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA) and  $1,25(\text{OH})_2\text{D}$  (by radioreceptor assay). Calcium and creatinine were measured in three successive 24-hour urine collections (Days 1 to 3). On Day 4, a 2-h fasting urine collection was obtained for measurement of calcium and creatinine, and a 4-h urine collection was obtained for the same tests after an oral ingestion of a synthetic meal containing 1 g of calcium. The calciuric response after the calcium load gave an indirect measure of intestinal calcium absorption (1,3). Fractional calcium absorption ( $\alpha$ ) was determined either from the fecal recovery of  $^{47}\text{Ca}$  after ingestion of a synthetic test meal containing trace radiocalcium (2), or by using a double stable isotope technique (12). The two tests yielded equivalent results. BMD of L2-L4 vertebrae was measured using dual energy x-

ray absorptiometry (Hologic QDR-2000, Waltham MA). Z score indicated deviation from the age- and sex-matched control value expressed in standard deviation. T score represented deviation from the normal peak value expressed in standard deviation. T score of less than -2.5 is defined as osteoporosis. A heparinized venous blood was obtained for lymphocyte isolation and immortalization and an EDTA treated venous blood for genomic DNA isolation (13).

Some patients underwent an outpatient evaluation (1) following one week on an instructed diet designed to mimic the inpatient metabolic diet in sodium, calcium and phosphorous content. This evaluation included: a fasting venous serum for calcium, creatinine, iPTH and  $1,25(\text{OH})_2\text{D}$  determination, a heparinized venous blood sample for lymphocyte isolation and immortalization, an EDTA treated venous blood sample for genomic DNA isolation, a 24-hour urine collection for calcium and creatinine determination, a 2-h fasting urine collection for calcium and creatinine determination, and a 4-h urine collection for the same tests after an oral ingestion of a synthetic meal containing 1 g of calcium (1,3). In addition, BMD was measured as noted in the previous section.

Normal volunteers: Normal volunteers (n= 132) were recruited from hospital and laboratory staff. All participants completed a standardized questionnaire detailing personal and family history of kidney stone formation and osteoporosis. A venous blood sample was obtained for lymphocyte transformation and DNA isolation, and for the measurement of serum iPTH,  $1,25(\text{OH})_2\text{D}$  and calcium. Any volunteer with personal or family history of stone disease or osteoporosis, or an abnormal serum PTH or calcium, was excluded from the study.

cDNA Sequencing. The nucleotide sequence of the full length cDNA encoded by the AH gene was determined as follows. Normal human intestinal cDNA (Marathon ready cDNA) and normal human intestinal mRNA was purchased from Clontech (Palo Alto, CA). Rapid amplification of cDNA ends (RACE) was used to obtain 5' and 3' sequence data. RACE ready

cDNA was prepared from the intestinal mRNA using a RLM RACE kit from Ambion (Austin, TX) in accordance with the supplier's instructions. Similarly, RACE analysis was performed directly on the Marathon ready cDNA using a SMART RACE cDNA amplification kit (Clontech). Forward and reverse primers (Table 1) were designed, based on the cDNA sequence of the hypothetical protein (Gen Bank accession AL035122) or on predicted exon sequence obtained by analysis of the genomic PAC clones dj313L4 and 295C6 using the gene finding program GRAIL (14). DNA sequence analysis was performed using an ABI Big Dye cycle sequencing kit (Applied Biosystems, Foster City, CA.) and analyzed on an ABI 377 automated DNA sequencer. Intron exon boundary information was obtained by alignment of the cDNA sequence with the genomic PAC clones dj313L4 and 295C6 using the BLASTN 2.1.1 computer program at NCBI.

**Multiple Tissue Array Screening.** A human multiple tissue expression array (Clontech, Palo Alto, CA) was screened using a 526 base pair probe which spanned exons 17–20 of the AH cDNA. The probe was generated by PCR using the following primers - 2168F :5'-tggattcgaggctctggagat and 2694R: 5'-aagtctcatgctatccagctggatc. Prior to hybridization the probe was labeled with digoxigenin-dUTP for chemiluminescent detection with CSPD according to the manufacturers protocol (Boehringer Mannheim, Indianapolis, IN). Hybridization was carried out under stringent conditions using the standard protocol recommended for chemiluminescent detection. Following exposure and development, the negative was scanned prior to digital quantitation. After the initial hybridization, the blot was stripped and reprobed with a control ubiquitin probe (Clontech) labeled and detected as described above.

**Mutation Screening. PCR and DNA Sequencing.** Genomic DNA was prepared from peripheral blood lymphocytes using a whole blood DNA extraction kit (Qiagen, Chatsworth, CA.). An initial screening for mutations was performed by sequencing all 33 exons in the



genomic DNA from 16 AH patients. Primers were designed based on the DNA sequence from each flanking intron. PCR amplification was performed in a total volume of 50  $\mu$ l with 50- 100 ng DNA, 2.0 U AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp, Norwalk, CT), 50 pmol of each primer, 1XPCR buffer (Perkin-Elmer Corp.), 200  $\mu$ M dNTP,  $MgCl_2$  was between 1-2.5 mM as specified for individual primer sets. Amplification conditions were as follows: 10 min initial denaturation at 95 $^{\circ}$ , followed by 35 cycles of (10 seconds at 95 $^{\circ}$ , 30 second annealing at between 58 $^{\circ}$ -68 $^{\circ}$  as specified for individual primer sets, 45 second at 72 $^{\circ}$ ) with a final 10 min incubation at 72 $^{\circ}$ . All primer sets used to amplify genomic DNA are depicted in Table 2.

Genomic DNA from 132 control subjects and 80 AH patients was examined for all mutations or polymorphisms revealed in the initial mutation screen. Since certain base changes destroyed a restriction endonuclease recognition site, restriction fragment polymorphism analysis was performed on the following PCR fragments amplified from genomic DNA of study participants: Bcg 1 (exon 7), Alu 1 (exon 11 and intron 23), Hae III or Mbo II (exon 20) (New England Biolabs, Beverly, MA.). All restriction enzyme digests were performed using the supplier recommended protocol. Fragment analysis was performed by electrophoresis on 1.5% agarose gels or 4% NuSieve (FMC Bioproducts, Rockland, Maine) when the resultant product size was < 100 base pairs in length. All mutations detected were verified by DNA sequence analysis.

**Statistical Analysis.** Allele frequencies were calculated for each genotype and the significant difference in allele frequencies between the AH and normal control populations was assessed using a Fisher's Exact test. Odds ratios for both the mutant alleles, as compared to the wild type, were calculated as a measure of the association between the mutant genotype and AH disease. These odds ratios were used as an estimate of relative risk. The association of mutation with bone density (L2-L4 BMD, Z score) was determined by multiple regression

analysis with correction for body mass index (BMI). Significance was assessed using a two-tail t-test, since variance between groups was equal. Difference in the number of patients meeting the criteria for osteoporosis (T score < -2.5) was assessed using a Fishers Exact test. In all analyses, a  $p < 0.05$  was considered significant. Statistical analyses were performed using SAS version 8.0 software.

## Results

**Characterization of AH Patients.** A total of 80 patients with AH were evaluated (63 men and 17 women)(Table 3). The mean age of the group was 48 years. All patients had the key features of absorptive hypercalciuria Type 1, including hypercalciuria on a restricted calcium diet, exaggerated calciuric response to an oral calcium load and/or elevated intestinal calcium absorption (1-3). All patients were normocalciemic and had normal or low serum iPTH. Included in this group were the three probands from our original linkage study (11).

**cDNA Sequence Analysis.** 5' and 3' cDNA fragments were generated by RACE using the primers 1921R and 1896 F. Subsequent primers were designed based on the 5' and 3' termini which allowed generation of the complete a 5085 base pair cDNA (Gen Bank accession #AF331033 ). Sequenced fragments were aligned and an open reading frame was predicted using the sequence navigator program (Applied Biosystems, Foster City, CA). The predicted protein contained 1518 amino acids (MW 176.5 kD) and a predicted pI of 7.61. The cDNA was coded by 33 exons with a corresponding gene size of 104 k bases. Molecular modeling predicted one guanylate cyclase catalytic subunit and two 34 amino acid tetratricopeptide-like regions (15). The AH gene cDNA and predicted protein were homologous to the human (GenBank accession AF176813) and rat soluble adenylate cyclase genes (16).

**Tissue Distribution and Sequence of mRNA.** Screening of a human multiple tissue array with a 526 base pair probe complementary to exons 17-20 of the AH gene indicated expression in several human tissues including kidney and jejunum (Fig. 1). The mRNA of the AH gene was also detected in human bone by RT-PCR (Fig. 2).

**Mutation Analysis.** Genomic DNA from the 3 probands of our original study kindreds (11) and 13 other AH patients with a family history of AH was initially sequenced. This task revealed 6 point mutations or polymorphisms. (a) In exon 7, c. 923 C→T changed a threonine to a methionine residue and destroyed a Bcg 1 restriction site. (b) In intron 11, c.1438+ 26 T→C destroyed an Alu 1 restriction site, (c) In intron 19, c.2667-20 C→T destroyed a Hae III restriction cleavage site. (d) A silent mutation within exon 20, c.2787 G→A destroyed a Mbo II restriction site. As the original hypothetical protein (Gen Bank accession AL035122) contained an alternate exon coded by a portion of intron 23, we screened this region. Two point mutations (e,f) were found separated by 70 bases, c.3531-580 A→T and c. 3531-507 G→T which destroyed an Alu I restriction cleavage site. The position of these base changes is indicated in Fig. 3.

The frequency of all 6 base changes disclosed in the AH group was compared with that of a normal population (Table 4). Two base changes were mutations with an allele frequency of <1% in the normal population. The c.3531-580 mutation was not found in 272 normal chromosomes screened while 7 mutations were found among 160 AH chromosomes. For the c.2787 G→A mutation, only 1 mutant allele was found out of 272 normal chromosomes screened, while 3 mutations were found out of 160 AH patient chromosomes. The remaining 4 base changes were polymorphisms as they occurred in >1% of the normal population as indicated in Table 4. The distribution of all 4 polymorphisms occurred with marginally or significantly elevated frequency in the AH population as compared to the control population.

The distribution of all polymorphisms was shown to be in Hardy Weinberg equilibrium in the control population. Nineteen additional base changes were found on screening the control population; most of these were common polymorphisms with a high frequency in the normal population.

**Human AH Genotype and Risk for AH.** The presence of the c. 923 C→T polymorphism in one or both alleles was associated with a significant 3.1-fold increase in estimated risk for stone formation and occurrence of the AH phenotype (hypercalciuria, elevated intestinal calcium absorption) (95% CI, 1.33-7.12). Similar calculation of the odds ratio for c.1438+ 26 T→C, c.2667-20 C→T and c.3531-580 A→T revealed an increase in estimated risk of 3.2 (95% CI, 1.36-7.52), 2.3 (95% CI, 1.19-4.27) and 2.63 (95% CI, 1.43-4.82) for one or both base substitutions of each mutation, respectively (Table 5).

**Association of Mutation/Polymorphism with L2-L4 BMD.** Since low bone density is frequently found in AH (7) and was disclosed in 3 probands of our original study kindreds (11), we examined the association between L2-L4 BMD and presence of 6 polymorphisms and mutations, (a) c. 923 C→T, (b) c.1438+ 26 T→C, (c) c.2667-20 C→T, (d) c.2787 G→A, (e) c.3531-580 A→T and (f) c.3531-507 G→T. L2-L4 vertebral BMD (Z score) was compared between 35 AH patients without any mutation or polymorphism and 45 patients with one or more mutations or polymorphisms. While mean BMD was not significantly different for the whole group (Table 3) there was a trend towards decreasing vertebral BMD with increasing number of mutations or polymorphisms (Fig. 4). The difference from the group without base changes was significant for the group with at least 3 or at least 4 base changes. For patients with at least 4 base changes, the decline in L2-L4 BMD was 2-fold greater than in patients without base changes (Z score  $-1.79 \pm 0.85$  vs  $-0.89 \pm 1.00$ ,  $p = 0.006$ ). The significant differences were maintained following correction for BMI. In addition, BMD corrected for

BMI was significantly lower among those with 3 or more mutations than in those with 0-2 mutations ( $p < 0.02$ ).

Eleven of the 45 AH patients with at least one mutation or polymorphism had L2-L4 BMD so low that they were classified as osteoporotic (defined as T score  $< -2.5$ ). In contrast, among AH patients without mutation or polymorphism, only 2 of 33 patients had T score of less than  $-2.5$ . The difference between the two groups was significant (Fig. 5,  $p = 0.006$ ).

**Association of Base Changes with Other Biochemistry.** Data from 35 AH patients without any base change were compared with those from 45 patients with at least one base change. There were no significant differences in age, height, weight, serum alkaline phosphatase, iPTH, calcitriol, urinary calcium, or intestinal calcium absorption.

## Discussion

In this report we have sequenced a candidate AH gene within the chromosome 1q 23.3–24 region previously identified by linkage analysis (11). Twenty-five known genes mapped to this interval none of which were candidates for the AH gene defect, hence a new gene encoding a hypothetical protein (Gen Bank accession AL035122) was chosen as the initial target in the search for the AH gene. This gene was comprised of 33 exons which encompassed a chromosomal region of approximately 104kb. In this "AH gene," 6 base changes were found predominantly or uniquely among patients with non-dietary dependent AH. The presence of four of these base changes, c.923 C→T, c.1438+26 C→T, c.2667-20 C→T, and c.3531-580 A→T, was shown to significantly increase the relative risk for AH. In addition a strong relationship was found between vertebral bone loss and occurrence of these mutations or polymorphisms.

Approximately 50% of index patients with AH were found to have mutations or polymorphisms. Some of the polymorphisms could be mutations, since the distribution of AH-associated alleles in the normal population may have been overestimated. It is known that the incidence of stone formation is 5-12% of the general population in the western hemisphere (17). Among reported cases of stone disease, about one-half can be ascribed to AH (1). Hence, although our healthy normal volunteers were neither stone-formers nor had a family history of stone formation, some of them could have had hypercalciuria.

Low bone density is a well-established complication of AH (7). We found that patients with AH who had mutations or polymorphisms in the AH gene resulting in the base changes (c. 923 C→T, c.1438+ 26 T→C , c.2667-20 C→T, c.2787 G→A, c.3531-580 A→T and c.3531-507

G→T) had a more prominent decline in L2-L4 BMD than those without these changes. This work suggesting an important putative role of the AH gene, is supported by other studies. In mice, a linkage was reported between bone density and a region on chromosome 1q23 which includes the AH gene (18). The genes in this region of mouse chromosome 1 closely corresponded to the location of the genes on human chromosome 1 (19). Furthermore, a human linkage study has shown an association between a gene or genes related to vertebral bone density and a broad region on chromosome 1q (20).

The exact biochemical mechanism by which mutations or polymorphisms in the AH gene produce the clinical sequelae of AH, such as low bone density, is not known. However, studies in rats suggest that the AH gene may code for a unique soluble adenylyate cyclase (sAC) in rat testis (16). While we were independently elucidating the sequence for the AH gene in human subjects, Buck et al. reported a soluble adenylyate cyclase at the same chromosomal locus with corresponding human gene posted to GenBank (Accession AF176813). The AH gene and sAC are quite similar. However, the cDNA from human intestine have several differences, most notably an inclusion of an additional 37 bases at the beginning of exon 5. This inclusion changes the reading frame prior to this point and inserts a new ATG or start codon at position 499 in the cDNA. Both encoded proteins beyond this point appear identical. An additional 20 bases were noted at the 3' terminus in the intestinal cDNA. They may represent a more complete sequence obtained by the RACE procedure. Several additional base changes noted may represent polymorphic sites. The protein predicted from the AH gene may be membrane bound as several weak membrane loops are predicted.

Although a role for sAC to date has only been described in male germ cells (21), a functional role in other tissues cannot be precluded. A recent study has proposed that the mammalian sAC acts as a bicarbonate sensor (22). This regulatory mechanism would be expected to keep the expressed constitutive activity of this enzyme low. The role of cAMP,

formed after the activation of the membrane bound adenylate cyclase, is well established in the regulation of calcium transport (23). It is tempting to speculate that mutations in the AH gene may cause an increase in the expressed adenylate cyclase activity of the gene product in intestine and that the resulting increase in intracellular cAMP leads to the increased calcium absorption. Recent studies have shown that PTH regulation of osteoclast differentiation factor (ODF) occurs via the protein kinase A/cAMP pathway in murine stromal cells (24). Hence, in bone upregulation of an adenylate cyclase could also lead to increased osteoclastogenesis with resultant increase in bone resorption.

It is noteworthy that only one-half of the patients with AH revealed mutation or polymorphism in the AH gene. Thus, other mutations or acquired factors must be invoked. In some patients, acquired causes have been invoked for the intestinal hyperabsorption of calcium, hypercalciuria and bone loss (25,26). This scheme could account for a mild low bone mass among AH patients without base change in the AH gene. Concerning genetic etiology, only one study has previously described a chromosomal lesion associated with absorptive hypercalciuria, namely a large 4q deletion (27). However since this region covered many genes, it is difficult to determine whether hypercalciuria was a direct result of a specific gene deletion or resulted secondarily from effects on another gene. Prior studies have sought aberrations in either vitamin D metabolism or the vitamin D receptor (VDR), since the central pathophysiological defect in AH is excessive intestinal calcium absorption (28-31). However, no linkage between the AH phenotype and either the 1-alpha-hydroxylase (the terminal enzyme of the vitamin D synthetic pathway) gene locus or the VDR was disclosed (32), and the VDR sequence was found to be normal (33). Other studies designed to determine a causative factor for AH have focussed on the Ca/ATPase (34) or an abnormality of bone metabolism related to elevated cytokines (35,36). Again, no genes related to these putative mechanisms reside in the chromosomal locus we have identified (11). Genetic studies based upon linkage



analysis and positional cloning have previously been successful in finding the gene for another disease marked by hypercalciuria. Dent's disease has been shown to be due to mutations in the chloride channel gene, *ClCN5* (37). However, most patients with AH do not share the full clinical manifestation of Dent's disease.

While the exact metabolic consequences of AH gene mutations remain to be determined, a key role in the regulation of calcium homeostasis in intestine and bone is strongly indicated.

## References.

- 1) Pak, C.Y.C., Britton, F., Peterson, R., Ward, D., Northcutt, C., Breslau, N.A., McGuire, J., Sakhaee, K., Bush, S., Nicar, M., et al. 1980. Ambulatory evaluation of nephrolithiasis: classification, clinical presentation and diagnostic criteria. *Am. J. Med.* 69: 19-30.
- 2) Pak, C.Y.C., Ohata, M., Lawrence, E.D., and Snyder, W. 1974. The hypercalciurias: causes, parathyroid functions and diagnostic criteria. *J. Clin. Invest.* 54:387-400.
- 3) Pak, C.Y.C., Kaplan, R., Bone, H., Townsend, J., and Waters, O. 1975. A simple test for the diagnosis of absorptive, resorptive and renal hypercalciurias. *New Engl. J. Med.* 292: 497-500.
- 4) Preminger, G.M., Peterson, R., and Pak, C.Y.C. 1989. Differentiation of unclassified hypercalciuria utilizing a sodium cellulose phosphate trial. In: *Urolithiasis*. Walker, V.R., Sutton, R.L, Cameron, E.C.B., Pak, C.Y.C., and Robertson, W.G. editors: New York and London: Plenum Press. 325-328.
- 5) Barkin, J., Wilson, D.R., Manuel, M.A., Bayley, A., Murray, T. and Harrison J. 1985: Bone mineral content in idiopathic calcium nephrolithiasis *Min. Electrol. Metab.* 11: 19-24.
- 6) Bataille, P., Achard, J.M., Fournier, A., Boudailliez, B., Westeel, P.F., Esper, N.E., Bergot, C., Jans, I., Lalau, J.D., Petit, J., et al. 1991. Diet, vitamin D and vertebral mineral density in hypercalciuric calcium stone-formers. *Kidney Int.* 39:1193-1205.
- 7) Pietchmann, F., Breslau, N.A., and Pak, C.Y.C. 1992. Reduced vertebral bone density in hypercalciuric nephrolithiasis. *J. Bone Min. Res.* 7:1383-1388.
- 8) Pak, C.Y.C. 1979. Physiological basis for absorptive and renal hypercalciurias. *Am. J. Physiol.* 237:F415-F423.
- 9) Coe, L., Parks, J.H., and Moore, E.S. 1979. Familial idiopathic hypercalciuria. *New Engl. J. Med.* 300:337-340.

- 10) Pak, C.Y.C., McGuire, J., Peterson, R., Britton, F., and Harrod, M.J. 1981. Familial absorptive hypercalciuria in a large kindred. *J. Urol.* 126: 717-721.
- 11) Reed, B.Y., Gitomer, W.L., Heller, H.J., Gitomer WL, and Pak, C.Y.C. 1999 . Mapping a gene defect in absorptive hypercalciuria to chromosome 1q23-q24. *J. Clin. Endocrinol. Metab.* 84: 3907-3913.
- 12) Abrams, S.A. 1994. Clinical studies of mineral metabolism in children using stable isotopes. *J. Ped. Gastroenterol. Nutr.* 19: 151-163.
- 13) Neitzel, H. 1989. A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum. Genet.* 73: 320-326.
- 14) Mural, R.J., Einstein, R., Guan, X., Mann, R.C., and Uberbacher, E. C. 1992. An artificial intelligence approach to DNA sequence feature recognition. *Trends Biochem.* 10:66-69.
- 15) Bateman, A., Birney, E., Durbin, R., Eddy, S.R., Finn, R.D., and Sonnhammer, E.L.L. 1999. Pfam 3.1: 1313 multiple alignments match the majority of proteins, *Nucleic Acids Research* 27:260-262
- 16) Buck, J., Sinclair, M.L., Schapal, L., Cann, M.J., and Levin, L.R. 1999. Cytosolic adenylate cyclase defines a unique signaling molecule in mammals. *Proc. Natl. Acad. Sci.* 96: 79-84.
- 17) Johnson, C.M., Wilson, D.M., O'Fallon, W.M., Malek, R.S., and Kurland, L.T. 1979. Renal stone epidemiology: A 25-year study in Rochester, Minnesota. *Kidney Intern.* 16: 624-631.
- 18) Klein, R.F., Vartanian, K.V., Skinner, L.D., Turner, R.J., Pelz, G.B., Carlos, A.S., Belknap, J.K., Shea, M., and Orwoll, E.S. 2000. Bone size and density are regulated by distinct chromosomal regions. *J. Bone Min. Res.* 15 (suppl 1) : S186. (Abstr.)
- 19) Blake, J.A., Richardson, J.E., Davisson, M.T., and Eppig, J.T. 1999 . The Mouse Genome Database (MGD): genetic and genomic information about the laboratory mouse. The Mouse Genome Database Group. *Nucleic Acids Research.* 27: 95-98.

- 20) Koller, D.L., Econs, M.J., Morin, P.A., Christian, J.C., Hui, S.L., Parry, P., Curran, M.E., Rodriguez, L.A., Conneally, P.M., Joslyn, G., et al. 2000. Genome screen for QTL's contributing to normal variation in bone mineral density and osteoporosis. *J. Clin. Endocrinol. Metab.* 85: 3116-3120.
- 21) Sinclair, M.L., Wang, X-Y., Mattia, M., Conti, M., Buck, J., Wolgemuth, D.J., and Levin, L.R. 2000. Specific expression of soluble adenylate cyclase in male germ cells. *Mol. Reprod. Dev.* 56: 6-11.
- 22) Chen, Y., Cann, M.J., Litvin, T.N., Iourgenko, V., Sinclair, M.L., Levin, L.R., and Buck, J. 2000. Soluble adenylate cyclase as an evolutionarily conserved bicarbonate sensor. *Science* 289: 625-628.
- 23) Gilman, A.G. 1984 . Proteins and dual control of adenylate cyclase. *Cell* 36:577-579.
- 24) Kondo, H., Guo, J., and Bringhurst, F.R. 2000. PTH regulates expression of ODF and OPG by different signaling pathway in murine marrow stromal cells. *J. Bone Min. Res.* 15 (suppl 1) : S445. (Abstr.)
- 25) Breslau, N.A., McGuire, J.L., Zerwekh, J.E. and Pak, C.Y.C. 1982. The role of dietary sodium on renal excretion and intestinal absorption of calcium and on vitamin D metabolism. *J. Clin. Endocrinol. Metab.* 55: 369-373.
- 26) Breslau, N.A., Brinkley, L., Hill, K.D., and Pak, C.Y.C. 1988. Relationship of animal protein-rich diet to kidney stone formation and calcium metabolism. *J. Clin Endocrinol. Metab.* 66: 140-146.
- 27) Imamura, K., Tonoki, H., Wakui, K., Fukushima, Y., Sasaki, S., Yausda, K., Takekoshi, Y., and Tochimaru, H. 1998. 4q33-qter deletion and absorptive hypercalciuria: report of two unrelated girls. *Am. J. Med. Genet.* 78: 52-54.

- 28) Insogna, K.L., Broadus, A.E., Dreyer, B.E., Ellison A.F., and Gertner, J.M. 1985. Elevated production rate of 1,25-dihydroxy D in patients with absorptive hypercalciuria. *J. Clin. Endocrinol Metab.* 61: 490-495.
- 29) Breslau, N.A., Preminger, G.M., Adams, B.V., Otey, J., and Pak, C.Y.C. 1992. Use of ketokonazole to probe the pathogenic importance of 1,25-dihydroxyvitamin D in absorptive hypercalciuria. *J. Clin. Endocrinol. Metab.* 75: 1446-1452.
- 30) Li, X.Q., Tembe, V., Horowitz, G.M., Bushinsky, D.A., and Favus, M.J. 1993. Increased intestinal vitamin D receptor in genetic hypercalciuric rats: a cause of intestinal calcium hyperabsorption. *J. Clin. Invest.* 91: 661-667.
- 31) Zerwekh, J.E., Yu, X-P., Breslau, N.A., Manologas, S., and Pak, C.Y.C. 1993. Vitamin D receptor quantitation in human mononuclear cells in health and disease. *Mol. Cell Endocrinol.* 9: 1-6.
- 32) Reed, B., Heller, H.J., Lemke, M., Lane, H., Reza Albarran, A., Zerwekh, J.E., Breslau, N.A., and Pak, C.Y.C. 1996 Linkage analysis in absorptive hypercalciuria: lack of linkage to the vitamin D receptor or 1,25(OH)<sub>2</sub>D<sub>3</sub> hydroxylase loci In: Pak CYC, Resnick M, Preminger G, eds. *Urolithiasis*. Dallas: Millet Press; pp 540-542.
- 33) Zerwekh J, Hughes, M R, Reed BY, et al 1995 Evidence for normal vitamin D receptor messenger ribonucleic acid and genotype in absorptive hypercalciuria *J Clin Endocrinol Metab.* 80: 2960-2965.
- 34) Bianchi G, Vezzoli G, Cusi, D, et al. 1988 Abnormal red-cell calcium pump in patients with idiopathic hypercalciuria. *N Engl J Med.* 319: 897-901.
- 35) Pacifici R, Rothstein M, Rifas L, et al. 1990 Increased monocyte interleukin-1 activity and decreased vertebral bone mineral density in patients with fasting hypercalciuria. *J Clin Endocrinol Metab.* 71: 138-145.

- 36) Weisinger JR, Alonzo E, Bellorin-Font E, Blasini AM, Rodriguez M.A., Paz-Martinez V, Martinis R. 1996 Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria Kidney Int. 49: 244-250.
- 37) Lloyd SE, Pearce SHS, Fisher SE, et al. 1996 A common molecular basis for three inherited kidney stone diseases. Nature. 379: 445-449.

**Table 1. Sequencing Primers used to Obtain Human Intestinal cDNA Sequence.**

UPM/NUP nested primers RACE primers (Ambion, Austin TX.)

AP1/AP2 nested primers (Clontech, Palo Alto CA)

380R: 5'-gtccatgtacatggcactgctt

208 F: 5'-tccaggactggcccatagtc, 790 R: 5'-agctgccagcagtttgggtga

619 F: 5'-caggagtgggaagaaggcctag, 1155 R: 5'-gcctatctcttctgctttgtctt

1073F: 5'-gctatttatctgagcttcgccca/1497F: 5'-gacctgcgactctgtcaccta, 1921 :R 5'-  
ggcctaggacattggccatgaacac

1684F: 5'-ggacgtaataaagagatcaactact, 2188 : R 5'-atctccaggaggtcgaatcca

1896 F: 5'-gcctaggacattggccatgaacac/ 2168 F: 5'-tggattcgacctctggagat,

2694 R: 5' aagtctcatgctatccagctggatc

2346 F: 5'-gatctgtcttgacctcaatgtgagagct, 2694 R: 5'-aagtctcatgctatccagctggatc

2450 F: 5'-acctggaacatcatgaggtactcg, 2860 R: 5'-gaagctccttgccattccggaaac

2670 F: 5'-gatccagctggatagcatgagactt, 2996 R: 5'-ctgtggcactcgatcacctcattctc

2837F: 5'-gtttccggaatggcaaggagcttcaa, 3676 R: 5'-tcttggcaagcactatctggc

3174 F: 5'-caacgcttttagacatggatggca, 3699 R: 5'-cttcagtgccttctcagcat

3590 F: 5'-cttgagccagacatttgagtctgccacc, 4034 R: 5'-cacacacctttgtagccagcca

3916 F: 5'-cacctggcagttatgatgcaaatga, 4489 R: 5'-aggaggagtccactgtggaacttc

4446 F: 5'-caacagaatcctcaagttccacagtggact, 4839 R: 5'-ccgcaaggctgtgttcaggaagaggcc

4813 F: 5'-ggcctcttctgaacacagccttgccg

Table 2. Sequence of Exon Specific Primers used to Amplify AH Gene and PCR Conditions.

Primer	MgCl <sub>2</sub> mM	Anneal at °C
Exon 1F: 5'- ATGAGCTACTGATGAGGGTGTG-3'	1.5	62 <sup>0</sup>
Exon 1R: 5'-ACTCCACAGCACCTTGCCAGA-3'		
Exon 2F: 5'- GAGCAATAGGAAAATGAGCAAATG-3'	2.0	63 <sup>0</sup>
Exon 2R: 5'- TACTCTGCAATCAAGCTCTGCAT-3'		
Exon 3F: 5'- TAGTCCTCTTCTGGCTCTGT-3'	1.5	60 <sup>0</sup>
Exon 3R: 5'- GTGCTAAGCAATTGTACTCTATCAG-3'		
Exon 4F: 5'- CTGAATCACTGTTCTTTTGACTAG-3'	1.5	55 <sup>0</sup>
Exon4R: 5'-ACTCCCTTCTCTAGGATGCCT-3'		
Exon 5F: 5'-AGGCATCCTAGAGAAGGGAGT-3'	1.5	55 <sup>0</sup>
Exon 5R: 5'- CTGAGCCACCATGTTCTACTA-3'		
Exon 6F 5'-TGCTCTGAGATAGTATAGTC-3'	1.5	55 <sup>0</sup>
Exon 6R 5'-TATGAAACCAGCGTGCCCAGT-3'		
Exon 7F: 5'-GCATACTCTCTTTCTGCTAAGA-3'	2.0	60 <sup>0</sup>
Exon 7R: 5'-ATGAAGCTGTCGGCATTGATATAAG-3'		
Exon 8F: 5'-TCTCACTAGCACTGCAGAGC-3'	1.5	55 <sup>0</sup>
Exon 8R: 5'-AGCTGTCCAGATCCCTGACA-3'		
Exon 9F:, 5'-ACCCATTCTGAGGTAGACATTC-3'	1.5	55 <sup>0</sup>
Exon 9R: 5'-CTATTCTCACCAATGTGCTTGT-3'		
Exon 10F: 5'- GCTCTCTGTTGAGGCCTTCTG-3'	1.5	55 <sup>0</sup>
Exon 10R: 5'- CAGTTTGAGACACAGGACTAAG-3'		
Exon 11F: 5'-CACTTTCTCTACGCTCATTATC-3'	2.0	60 <sup>0</sup>
Exon 11R: 5'-TCTGAGGGCAGGGCTCATGT-3'		



Exon 12F: 5'-CTTCTGTGTCACATGAACAGC-3'	2.0	60°
Exon 12R: 5'-TGAAGTCTGGGCCTTGGTCTG-3'		
Exon 13F: 5'-GATAGACTCTTGTTAGCAATTGAG-3'	2.0	55°
Exon 13R: 5'-GATAATTCATTAAGCTGCACAGTTA-3'		
Exon 14F: 5'-AGAAGGAGGGTTTCTATGTGACT-3'	2.0	58°
Exon 14R: 5'-ATCAGGATTCTATGGGTTCACAC-3'		
Exon 15F: 5'-AGAAGACAGAGCTTTCCTTCAAC-3'	1.5	60°
Exon 15R: 5'-CTAGCTAGTTACCTGAACATGGA-3'		
Exon 16F: 5'-GACCACTTATCTAACACATGAG-3'	1.5	55°
Exon 16R: 5'-ATGTGGTCTGAACTCCTCAAAC-3'		
Exon 17F: 5'-TACATATGCATACCCATACATGTGT-3' Exon	1.5	60°
17R: 5'-GTATGCAGATGTCGAGAGTTCAG-3'		
Exon 18F: 5'-GATGTATCTTCAATGTAAACAGATTGCCT-3'	1.5	62°
Exon 18R: 5'-AGCAGCCTGTGGAATAGCTCAT-3'		
Exon 19F: 5'-AGACAGGATAATGACTCAACTTTTCTTC-3' Exon	1.5	63°
19R: 5'-GAT TACAGGCCTGAGCCACTG-3'		
Exon 20F: 5'-TACTTCTTTTGACTTAGCTTGGCTGAG-3'	2.5	68°
Exon 20R: 5'-CAGGCAGTGGGCAACAGATCA-3'		
Exon 21F: 5'-TGATCTGTTGCCCACTGCCTG-3'	1.5	63°
Exon 21R: 5'-CTCCTTCTAGATCTTAGTCTCTAGATTTC-3'		
Exon 22F: 5'-TCTTGGGTAGACAGCAGATATC-3'	1.5	55°
Exon 22R: 5'-TCCCCTTGCATCTAAGAGGTC-3'		
Exon 23F: 5'-TCCTGATCTCAGATGATCCACCA-3'	1.25	60°
Exon 23R: 5'-CTTCCTTCTGGCTCTATGTTCTAT-3'		

Intron 23F: 5'-TGATTAGAAGCACAGCCTCAGTGC-3'	1.5	62 <sup>0</sup>
Intron 23R: 5'-CATCTAGGTTGCCTTACCCGAAGT-3'		
Exon 24F: 5'-GTGGTAACTGAAATGGCTCCTGCAT-3'	1.5	63 <sup>0</sup>
Exon 24R: 5'-TGCTGGTAAGCCATGGTGAGGAG-3'		
Exon 25F: 5'-TAGTCATTCAACCACCCTTCATGC-3'	1.5	63 <sup>0</sup>
Exon 25R: 5'-GTCAGAGCCAAACAGGAGTCCTTG-3'		
Exon 26F: 5'-ATAGTGAGGACACCATGTACCTGTGC-3'	1.5	63 <sup>0</sup>
Exon 26R: 5'-CACTAGGCTTTTCTTCCCAAATGA-3'		
Exon 27-28F: 5'-CTTTGCGTTCTGCCTCACTCTT-3'	1.5	63 <sup>0</sup>
Exon 27-28R: 5'-GAACCCAGAATACTCAATAATACGGCCT-3'		
Exon 29F: 5'-AGTGCTGGGATTACAATATCCAC-3'	2.0	62 <sup>0</sup>
Exon 29R: 5'-TAAGTCCACCAGCCAGAAACCTA-3'		
Exon 30F: 5'-AGATGATCACCCACACCTACT-3'	1.5	63 <sup>0</sup>
Exon 30R: 5'-CTGACACCCTTAAGCTGTCTCA-3'		
Exon 31F: 5'-TTCCAAGGATTCTATTCATCTCAAG-3'	1.5	63 <sup>0</sup>
Exon 31R: 5'-TAGCACAGGAAGTAGACAGAGA-3'		
Exon 32F: 5'-CTTGAAATGTTCCCAAATCATCTGT-3'	1.5	63 <sup>0</sup>
Exon 32R: 5'-TGTTAATGGCAGCCTGGTGAAAC-3'		
Exon 33F: 5'-CTAGTTTGGACAGCAAGATTCG-3'	1.5	63 <sup>0</sup>
Exon 33R: 5'-GTCTGTTTCTGTGTCTGGAACA-3'		

Table 3. Mean Biochemical and Physiological Characteristics of AH Patients

Parameter	AH Patients	Normal Range
Age years	48 ± 13 (80)	
Height m	1.72 ± 0.08 (79)	
Weight kg	85 ± 16 (78)	
<b>Serum</b>		
Alkaline Phosphatase (IU/L)	74 ± 27 (75)	30-140
iPTH (ng/L)	34 ± 15 (60)	10-65
1,25-(OH) <sub>2</sub> D (pmol/L)	95 ± 27 (38)	48-132
Ca (mmol/L)	2.35 ± 0.10 (79)	
<b>Urine</b>		
24-h Ca (mmol/day)	7.08 ± 2.02 (80)	< 5.0
Fasting Ca (mmol/mmol creatinine)	0.029 ± 0.022 (68)	< 0.027
Post Load Ca (GF)	0.07 ± 0.03 (68)	<0.05
<b>Intestinal Ca Absorption</b>		
α%	70 ± 10 (41)	40-60
<b>Bone Density</b>		
L2-L4		
BMD (g/cm <sup>2</sup> )	0.95 ± 0.12 (78)	
t-score	-1.41 ± 1.10	
t %	85.7 ± 11.1 (78)	

z-score	-1.03 ± 1.08	
z%	89.6 ± 11.3 (78)	

All values are presented as mean ± SD, with number of samples given in *parentheses*.

**Table 4.** Allele Frequencies for Various Mutations and Polymorphisms of the AH gene in Healthy Normal Control and AH Populations.

		Population Frequency					
		Control		AH			
Mutation	Allele	Alleles tested	%	Alleles tested	%	$\chi^2$	P*
c.923	T	234	96	141	89		
	C	10	4	17	11	6.79	0.013
c.1438-26	C	179	95	138	87		
	T	9	5	20	13	6.99	0.011
c.2667-20	C	244	90	133	83		
	T	28	10	27	17	3.93	0.053
c.2787	G	271	99.6	157	98		
	A	1	0.4	3	2	2.41	NS
c.3531-580	A	226	88	118	74		
	T	30	12	42	26	14.52	<0.001
c.3531-507	G	272	100	153	96		
	T	0	0	7	4	12.10	<0.001

\*Fishers Exact two-sided probability.

**Table 5. Association Between Genotype and Estimated Risk for AH.**

Mutation	Genotype	Control		AH		Odds Ratio	95% CI	P
		Total	#(%)	Total	# (%)			
c.923	CT or TT	122	10(8)	79	17(22)	3.07	1.33-7.12	0.01
c.1437+26	CT or TT	94	9 (10)	79	20(25)	3.20	1.36-7.52	0.008
c.2667-20	CT or TT	136	25 (18)	80	27(34)	2.18	1.19-4.27	0.014
c.3531- 580	AT or TT	128	27(21)	80	35(44)	2.58	1.43-4.81	0.002

Odds ratio could not be calculated for c.3494-507 as control population contained 0 mutant alleles. c.2787 odds ratio was non-significant.

**Table 6. Comparison of Mean Biochemical and Physiological Characteristics of AH Patients Grouped According to Presence or Absence of Mutations 923 C→T, c.1438+ 26 T→C, c.2667-20 C→T, c.2787 G→A, c.3531-580 A→T and c.3531-507 G→T.**

Parameter	With Mutation (n)	Without Mutation (n)
Age years	47 ± 15 (45)	48 ± 11 (35)
Height m	1.70 ± 0.14 (44)	1.75 ± 0.08 (35)
Weight kg	82 ± 14 (44)	90 ± 17 (34)
<b>Serum</b>		
Alkaline Phosphatase (IU/L)	69 ± 28 (41)	80 ± 24 (34)
iPTH (ng/L)	33 ± 14 (34)	36 ± 16 (26)
1,25-(OH) <sub>2</sub> D (pmol/L)	96 ± 34 (26)	94 ± 24 (12)
Ca (mmol/L)	2.35 ± 0.11 (45)	2.32 ± 0.08 (35)
<b>Urine</b>		
24-h Ca (mmol/day)	6.89 ± 2.32 (45)	7.31 ± 1.52 (35)
Fasting Ca (mmol/mmol creatinine)	0.31 ± 0.17 (38)	0.37 ± 0.31 (30)
Post Load Ca (mmol/mmol creatinine)	0.74 ± 0.23 (38)	0.88 ± 0.31 (30)
<b>Intestinal Ca Absorption</b>		
α%	69 ± 11 (30)	71 ± 7 (11)
<b>Bone Density</b>		
<b>L2-L4</b>		
BMD (g/cm <sup>2</sup> )	0.95 ± 0.14 (43)	0.97 ± 0.11 (35)

t-score	-1.51 ± 1.14,	-1.26 ± 1.04,
t %	84.0 ± 11.0 (43)	88.0 ± 11.0 (35)
z-score	-1.12 ± 1.13,	-0.89 ± 1.01 ,
z %	88.5 ± 11.8 (43)	91.0 ± 11.0 (35)

All values are presented as mean ± SD, with number of samples given in *parentheses*.



## Legends to figures.

### Fig.1

Relative tissue expression of the human AH mRNA. A multiple tissue expression array (Clontech) was hybridized with a 526 base pair fragment of AH cDNA labeled with digoxigenin-dUTP. Following detection the blot was reprobred with a ubiquitin standard probe. The intensity of the AH signal relative to ubiquitin was plotted for each tissue spot.

### Fig 2.

Human bone AH mRNA detected by RT-PCR. A 526 base pair fragment of human AH cDNA was amplified by PCR. Lane A represents approximately 100 ng template cDNA, lane B, 25 ng template. The expression of AH mRNA is low relative to the abundance of  $\beta$  actin in the same sample.

### Fig 3.

Diagram of the human AH gene. Exons are represented by vertical bars and introns by horizontal lines. The relative positions of the studied mutations and polymorphisms is indicated.

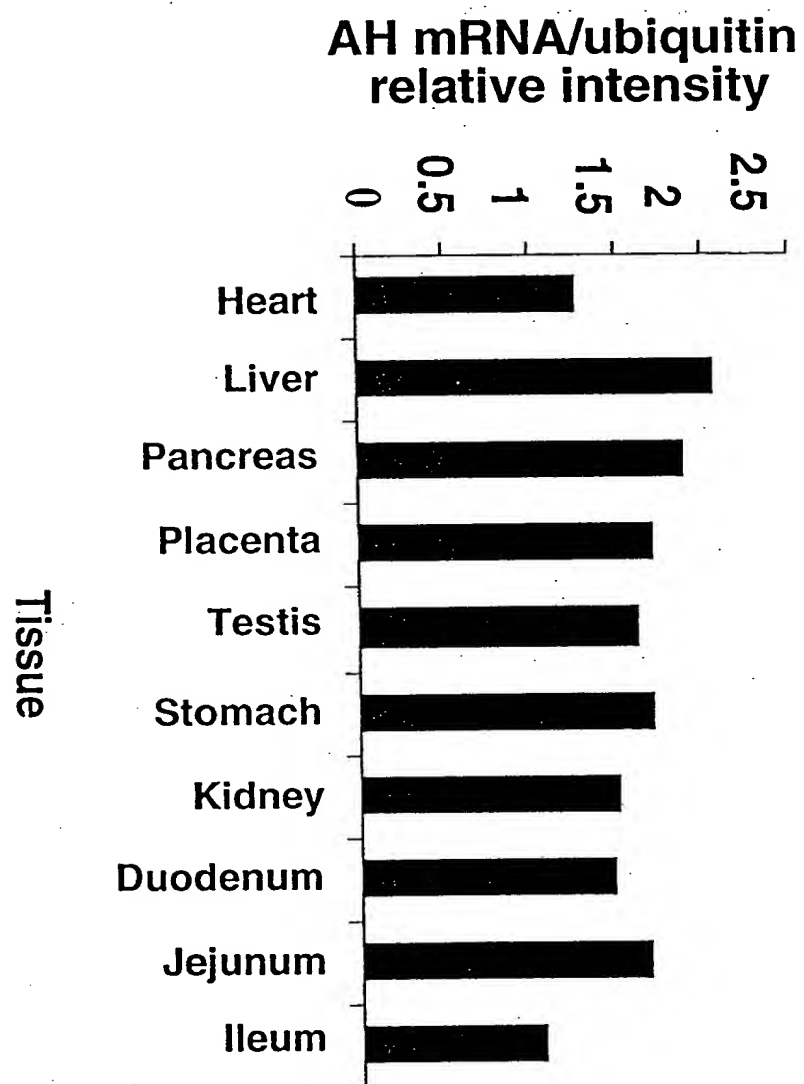
### Fig.4.

The association between lumbar (L2-L4) bone density and mutations or polymorphisms in AH patients. The y-axis indicates the Z score for L2-L4 BMD (# of standard deviation from the mean of age- and sex-matched normal BMD). On the x-axis, "0" represents AH patients without any base changes (n = 35), " $\geq 1$ " at least one base changes (n= 43), " $\geq 2$ " at least 2 base changes (n= 33), " $\geq 3$ " at least 3 base changes (n=16), and " $\geq 4$ " at least 4 base changes (n=13).

The mean Z scores are given at the bottom of the bars, and p value indicates the significant difference from "0". N refers to the number of patients in each group.

**Fig.5.**

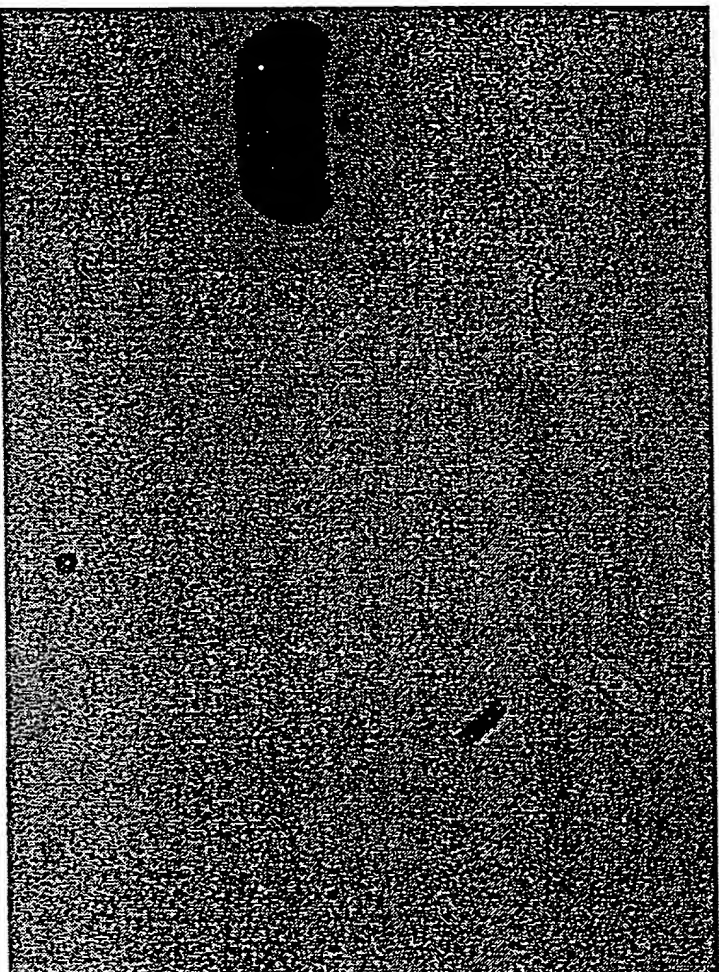
The prevalence of spinal osteoporosis among AH patients with and without base changes. The percentage of patients having T score for L2-L4 BMD of less than  $-2.5$  among AH patients without any base changes are compared with that of patients with at least one base change.



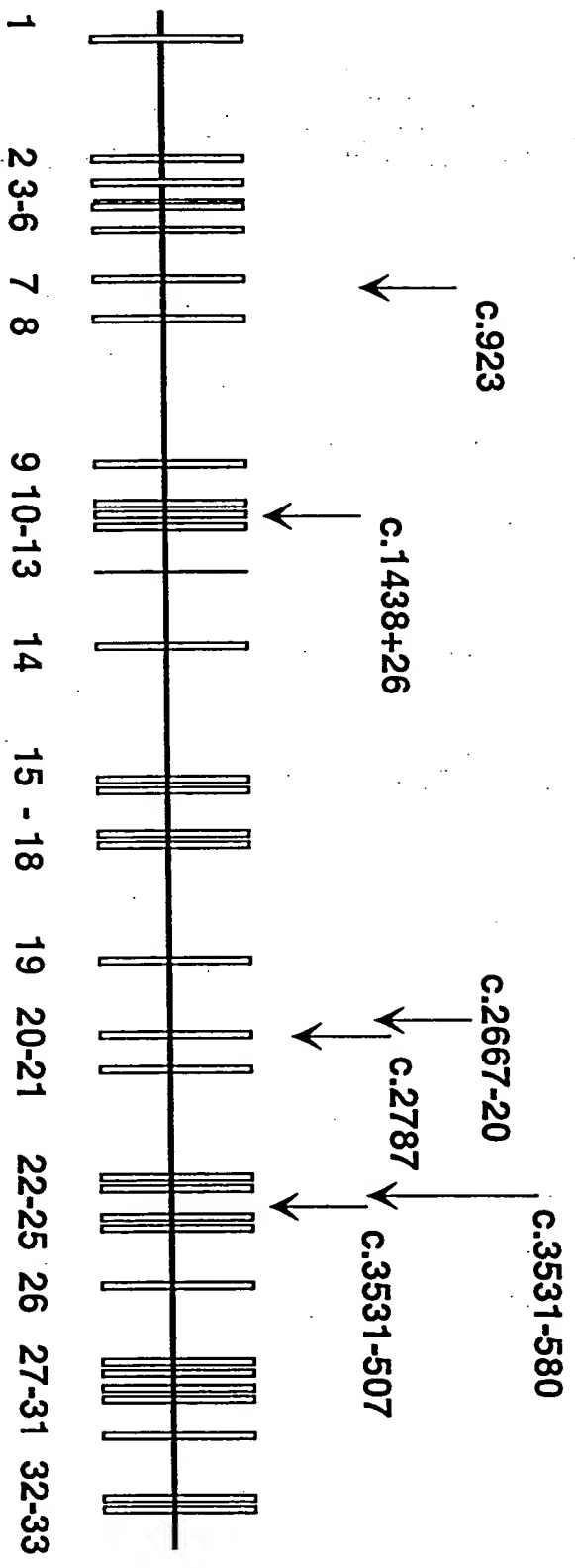
$\beta$ -actin

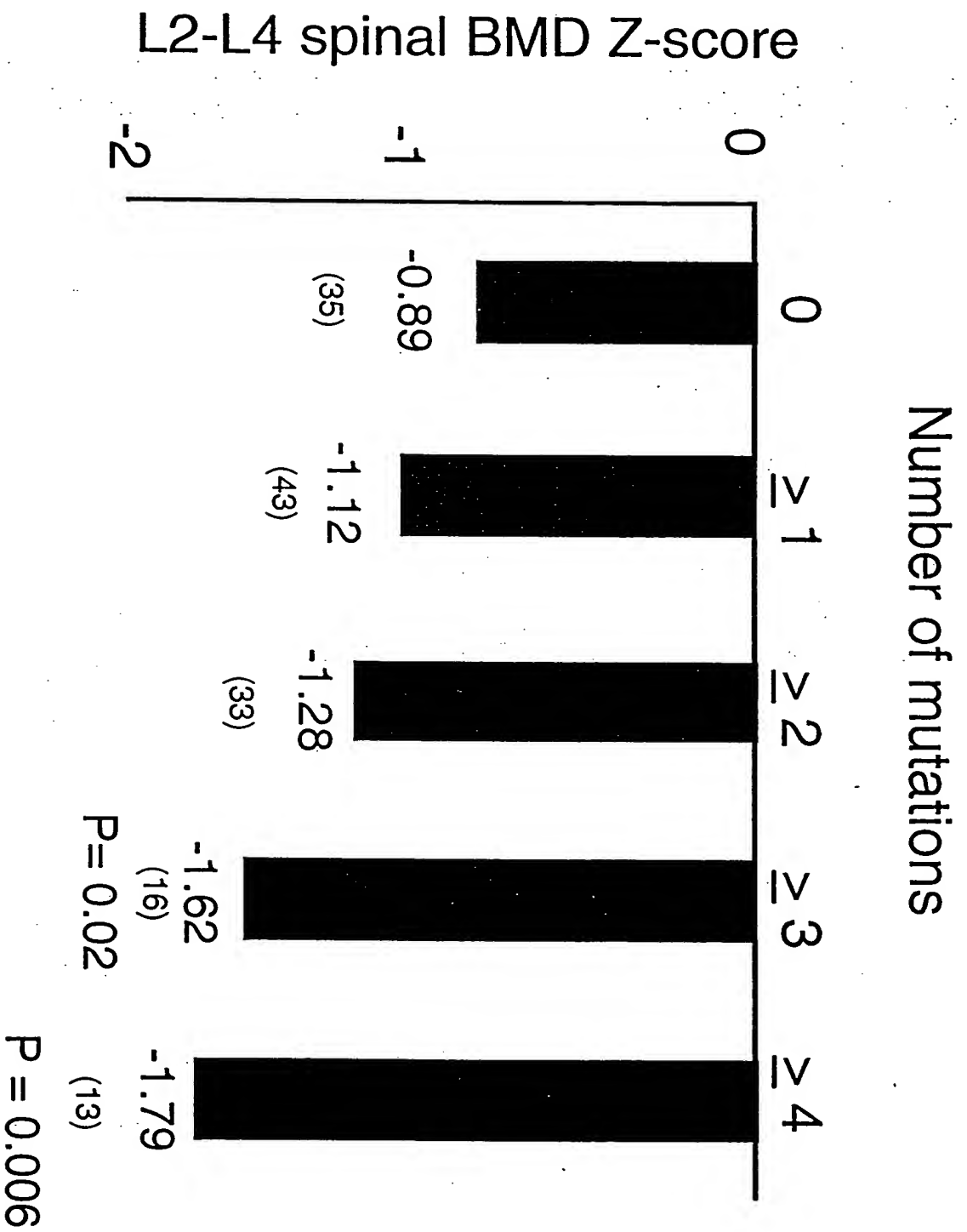
AH (a)

AH (b)

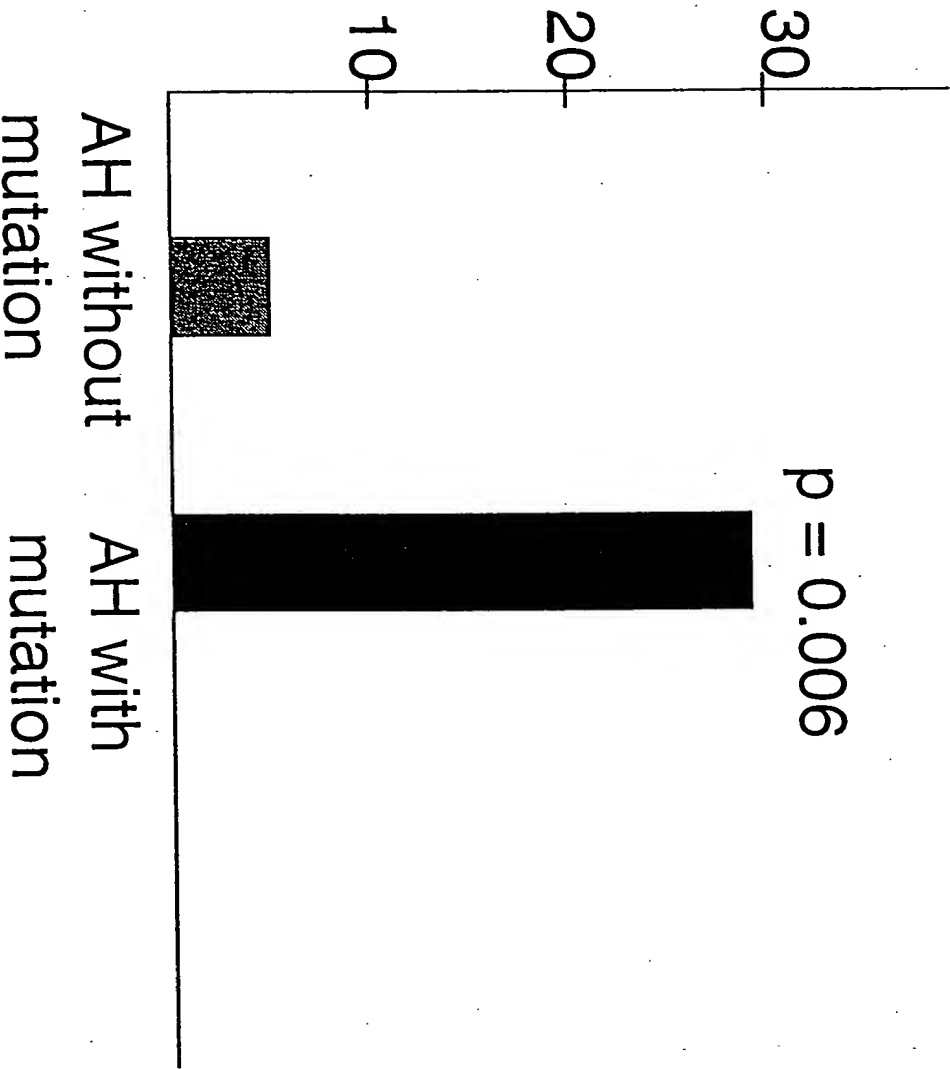


—526bp





% patients with L2-L4 T-score < -2.5



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Comment: Source human intestine

LOCUS nkit376922 5085 bp mRNA PRI 21-DEC-2000

DEFINITION Homo sapiens chromosome 1 map 1q24.

ACCESSION ;

KEYWORDS

SOURCE human.

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 5085)

AUTHORS Reed,B.Y., Gitomer,W.L., Heller,H.J., Hsu,M., Lemke,M.,  
Padalino,P.K. and Pak,C.Y.C.

TITLE Analysis of the human absorptive hypercalciuria gene and  
association of base changes with low spinal bone density

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 5085)

AUTHORS Reed,B.Y., Gitomer,W.L., Heller,H.J., Hsu,M., Lemke,M.,  
Padalino,P.K. and Pak,C.Y.C.

TITLE Direct Submission

JOURNAL Submitted (21-DEC-2000) Center for Mineral Metabolism and Clinical  
Research, UT. Southwestern Medical Center at Dallas, 5323 Harry  
Hines Blvd., Dallas, TX 75390-8885, USA

COMMENT Bankit Comment: Source human intestine.

FEATURES Location/Qualifiers

source 1..5085

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/db\_xref="taxon:9606"

/chromosome="1"

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Creation date: 08-01-2005  
Indexing Officer: NDINH3 - NGUYET DINH  
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